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The diatoms test in veterinary medicine: a pilot study on cetaceans and sea turtles.

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Highlights

- The application of the diatoms test in veterinary medicine is evaluated on marine vertebrates
- Diatoms were found in long bones of stranded cetaceans and sea turtles
- The protocol to assess the presence of diatoms in marine vertebrates was adapted and implemented

ABSTRACT

Fishing activities are considered one of the most relevant threats for cetaceans and sea turtles conservation since these animals are sometimes found dead entangled in fishing gears. Currently, postmortem diagnosis is based mainly on the presence of nets and lines on the body and the related marks and injuries evident at gross examination. A more detailed and objective evidence is needed to clarify doubts cases and the diatoms technique, used in forensic human medicine, could support drowning diagnosis also in this field. Diatoms' investigation was implemented to be applied in marine vertebrate on 8 striped (*Stenella coeruleoalba*) and 1 bottlenose (*Tursiops truncatus*) dolphins and 5 sea turtles (*Caretta caretta*) stranded along the Italian coastlines with a likely cause of death hypothized on necropsies carried out by veterinary pathologists. Diatoms were microscopically searched in the bone marrow collected from long bones implementing protocols used in human medicine and their presence was observed in 4 cetaceans and 2 sea turtles. Despite a clear relation between diatoms' presence and amount and the likely cause of death was not proved due to the poor number of samples, the higher burden of diatoms was found in 3 animals deemed to be death for the interaction with human activity. Despite more studied are necessary to identify the possible relation between the cause of death and diatoms' findings, the present study implemented this technique to be adapted to marine animals, confirming its possible application also in veterinary forensic medicine.

Keywords: diatoms, cetaceans, sea turtles, by-catch, drowning

INTRODUCTION

The most relevant causes of death of cetaceans are due to natural causes, to environmental changes, or can be caused by anthropogenic activities [1, 2, 3], including, but not limited to interaction with fishing activities [4, 5, 6], impact with acoustic sources [7], ship strikes [8, 9] and pathological agents and persistent chemical contaminants from domestic, industrial and agricultural wastes [10, 11].

Among the possible interactions with fisheries, the incidental capture and entanglement in fishing gears are frequently reported [12, 13], with gill-nets, drift-nets and trawls as the most frequent cause of by-catch for free-ranging odontocetes [13]. In these cases, marine mammals could drown because they cannot reach the surface and breath correctly. Furthermore, some individuals may accidentally ingest a hook, when they depredate catch from longline hooks [14, 15, 16]. These events may lead to internal injuries, infections, starvation, or even eventual death [17], but some hooked individuals may be unable to reach the surface to breathe, thus leading to a more immediate death by drowning [14]. Moreover, Gomerčić *et al.* in 2009 reported for the first time that ingestion of gill-net parts leads to larynx strangulation [3]. Similar considerations are reported also for sea turtles [18, 19].

In both taxa the interaction with fisheries is generally assessed during external evaluation and/or postmortem examinations, considering direct (i.e. presence of gears and lines, cutaneous linear and regular impression and external marks) and indirect (i.e. hemorrhages, fractures, blunt traumas, absence of extremities, pulmonary edema) evidences as well as the presence of fresh food remains in the stomach chambers and the absence of any other predisposing disease ongoing [9]. Scant information are related to the mechanism of death in order to support the final diagnosis according to an evidence-based approach, except for gross and microscopic pulmonary features related to asphyxia [9]. The aim of present study is to investigate the possible application of the research and counting of diatoms used in human forensic medicine to marine vertebrates and consider if drowning could be considered as a possible mechanism of death in these cases.

Diatoms are ubiquitous unicellular, eukaryotic microorganisms living in many different environments, included the marine environment. The hallmark of the diatoms is their cell wall, that is highly differentiated and almost always heavily impregnate with silica ($SiO_2 \cdot nH_2O$); they vary in size from 5 to more than 500 μm [20]. Diatoms, for their features (siliceous envelope) and distribution (specific species are related to specific environment) have been considered one of the more suitable indicator of drowning. They also may be found in moist soil and the atmosphere. The type of diatoms found in a certain location is unique and specific of that area. The season also affects what type is found. Diatoms can enter the body in three different ways: through inhalation of airborne diatoms, ingestion of material containing diatoms, and aspiration of water containing diatoms. This last route is the foundation for forensic diatom testing, which in conjunction with other findings provides a diagnosis of drowning [21, 22, 23]. Diatoms enter within the airways during water inspiration and they are able to pass in the circulatory system through the alveolar walls. In still alive animals, they are distributed to several tissues following the bloodstream and here diatoms remain intact thanks to their morphological features.

These algae are able to pass the air-blood barrier also in dead animals but they are not disseminated in different tissues through the circulation. In forensic human medicine, drowning diagnosis using diatom-test based on microscopic observation could be reached respecting specific criteria. More in detail a correspondence of diatoms amount, type and morphology between the drowned body and the water were it has been found should be ascertain [24, 25] with diatoms numbers in lungs and other organs which are respectively from 10 to 100 and from 100 to 1000 lower than into the water

[26]. Among all analyzed tissues, bone marrow has been considered as the most suitable due to the low contamination and influence of external and autolytic factors [24]. Slides obtained from bone marrow usually show less then 10 diatoms with an average size less than 30μm and an ovoidal or fusiform shape [21].

MATERIALS AND METHODS

In this preliminary study, bone marrow samples were collected from the humerus of 1 bottlenose dolphin (*Tursiops truncatus*) and 8 striped dolphins (*Stenella coeruleoalba*) since other long bones were not available due the evolutionary absence of rearlimbs. In order to obtain a comparable amount of tissue, both femur and the humerus were collected from 5 loggerhead sea turtles (*Caretta caretta*), due to the little size in these species. The bottlenose dolphin and the loggerhead sea turtles stranded along the Northern Adriatic coastline (Italy) whereas striped dolphins were found stranded along the Northern Tyrrenian shores (Liguria region, Italy) between November 2014 and March 2017.

During complete postmortem examinations carried out according to the national protocol based on international standards [27, 28], long bones were collected from the carcass by using scalpels. All the sampling and materials were accurately rinsed using fluids (distilled water or ethanol 96%) previously evaluated for the presence of diatoms by centrifugation and subsequent microscopic observation. The bones were fractured to sample bone marrow, according to protocols used in human medicine, which use from 5 to 50 cm^3 of tissue [21, 26]. In detail, 50 cm^3 were initially obtained according to Pollanen and collegues [21] from the first animal (the bottlenose dolphin) in order to assess the methodology, while in the other animals (8 striped dolphins and 5 sea turtles) 15 cm^3 were used according to internal routine procedures.

All the samples were incubated 24 hrs in a bottle of glass containing 2 volume of 70% HNO_3 per each volume of sample at the temperature of °50. Subsequently samples were refrigerated till lipids solidification. The liquid phase was then centrifuged (2500 rpm for 25 minutes); 100 μl of the obtained sediment was resuspended in 10 ml of distilled water and centrifuged again with the same parameters. 100 μl of the sediment was then put on slides and dried at °50. Diatoms were counted by microscopic observation at 400x magnitude.

RESULTS

The easiness in sampling and the good quality of the obtained material make the bones a suitable sampling site for this analysis. More in detail, sampling in striped dolphins cannot be carried out according to standard protocols due to the limited volume that could be obtained from their long bones (15 cm^3 in total, consisting of bone tissue and bone marrow): in fact, due to their specific features (i.e. reduced size and absence of the medullary cavity) bones had to be fractured and directly digested to collect properly the full amount of bone marrow.

In table 1, results of the diatoms-test carried out on the 9 stranded dolphins and 5 sea turtles under examinations have been summarized, grouping examined animals according to species (cetaceans and sea turtles), likely manner of death (natural vs accidental) [23], possible causes of death according to 2018 ICD-CM classification (www.cdc.gov/nchs/icd/icd10cm.htm) and their etiology. Positive results were reported in 4/9 cetaceans and in 2/5 sea turtles with the highest number found in the bottlenose dolphin deemed to be died for the interaction with human activity (fig. 1) and in a striped dolphin affected by dolphin morbillivirus (DMV): in these individuals, the amount of diatoms found in the bone marrow was respectively 2 and 3 orders of magnitude greater than in animals died respectively for fungal infection and toxoplasmosis. In all these three cases of spontaneous disease breathing control could be affected to damages related to central nervous system as well as to airways, as reported in table 1. Also for sea turtles, diatoms were found in higher number in those subjects deemed to be killed by fishing activities (fig. 2).

DISCUSSION

The interaction between marine vertebrates and fishing activities is a relevant menace for their conservation. Postmortem diagnosis is often supported by external evidences and eventually by the presence of fishing gears around the carcass. On the contrary, the absence of clear external findings consistent with this interaction do not exclude possible entanglement. In the case of marine mammals, Moore et al. (2013) [9] define the main pathological changes related to by-catch considering gross findings, but further diagnostic approaches are necessary to confirm the possible diagnosis. The present study was aimed to investigate the possible application of the diatoms-test used in human forensic medicine to marine animals as a support for the diagnosis of by-catch.

Diatoms were found in 6 out of the 14 examined marine vertebrates, but the limited number of examined animals and the variety of cases do not allowed to state a clear association between their quantity in the bone marrow of dolphins and sea turtles and the manner and causes of death. Large amounts of diatoms were more frequently observed in those animals suspected to be drowned consequently to an interaction with fishing activities (i.e. entanglement in fishing gears and lines), but they were also found in some dolphins died for natural conditions. More in detail, the reported diseases found during necropsies were associated to pathological changes that could have affect central control of the respiratory activities (DMV and *T. gondii*) or impaired normal breathing due to the severity of lesions (respiratory aspergillosis) allowing the entrance of sea water in the respiratory system during life.

In addition to this hypothesis, the biology of these species should be also considered to explain these findings. Currently, our knowledge on behavioral, anatomical and physiological adaptations of these species to the marine environment cannot explain completely and clearly how diatoms enter the bodies of these animals. Both cetaceans and sea turtles are voluntary breath-holding animals able to respond to prolonged hypoxic conditions [30, 31, 32]. More in detail, sea turtles are able to use completely their oxygen storages in the lungs and all tissues may become anaerobic with large changes in blood pH and carbon dioxide tolerance during prolonged dives; furthermore, the brain is able to function in the absence of oxygen [31]. On the other side, small odontocetes could dive several minutes, depending on the maximum depth reached, due to specific affinity of hemoglobin and myoglobin for oxygen and to the large blood storages in several organs and tissues [32, 33]. Despite also marine mammals are more resistant to low blood oxygen pressure and hypercapnic condition compared to other terrestrial mammals, breath-hold duration is affected by high blood carbon dioxide pressure [33]. No information regarding consequences of prolonged anoxic condition as those occurring for a forced diving due to by-catch: in particular, it is still not clear if any involuntary breath with opening of the upper airways and the subsequent entrance of water in the airways could occur in hypercapnic and/or hypoxic conditions or during agonic phases, with subsequent drowning.

In conclusion, this study confirm that diatoms enter the body of marine vertebrates if they drowned representing a possible valuable support in the diagnosis of interaction with human activities both in cetaceans and sea turtles to be used along with other findings reported during necropsies [9]. This evidence should be evaluate considering pathological findings, excluding other possible cause of death or diseases which could have affect respiration. Further investigations increasing the number of examined animals are necessary to relate postmortem findings and diatoms quantification in order to support the use of this technique as a possible tool in detecting by-catch. Thanks to this evidence-based approach, one of the most relevant menaces for cetaceans' and sea turtles' conservation could be really understood avoiding under- or overestimation and mitigation measures could be properly recommended.

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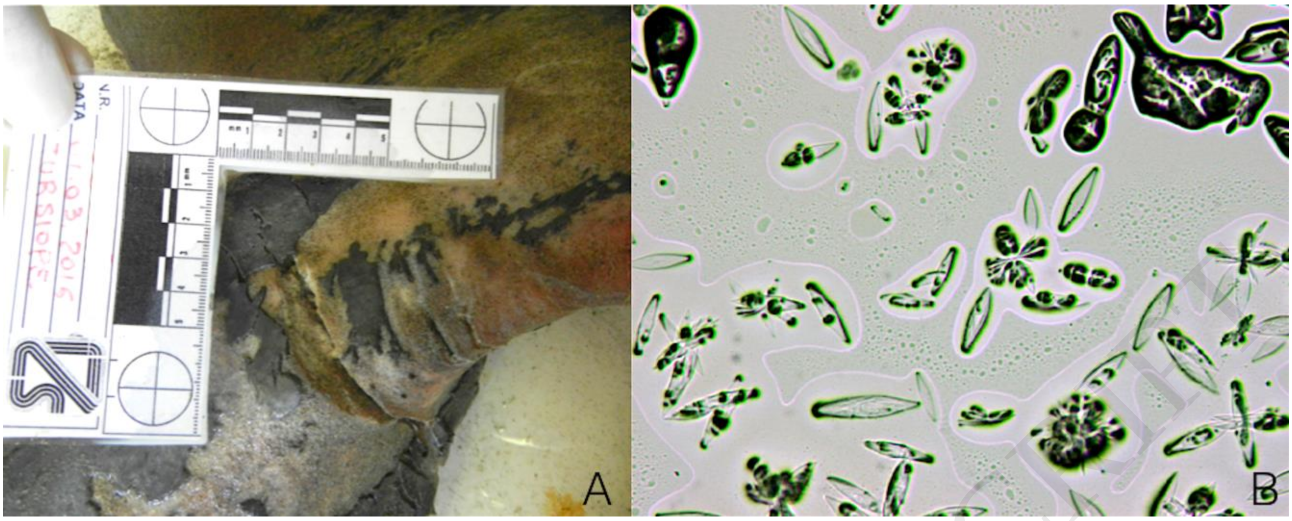

Fig. 1: diatoms in bottlenose dolphin ID 9.

In fig. 1A linear cuts to the tail are grossly evident and are suggestive of interaction with human activities: since fishing and/or killing of cetaceans is forbidden, fishermen who found a cetaceans died in their fishing gears often use a rope to sink the carcass; linear cuts could be also the results of freeing the nets from the by-caught animal; in fig. 1B microscopic evidences of diatoms in the bottlenose dolphin's no. 1 bone marrow are evident.

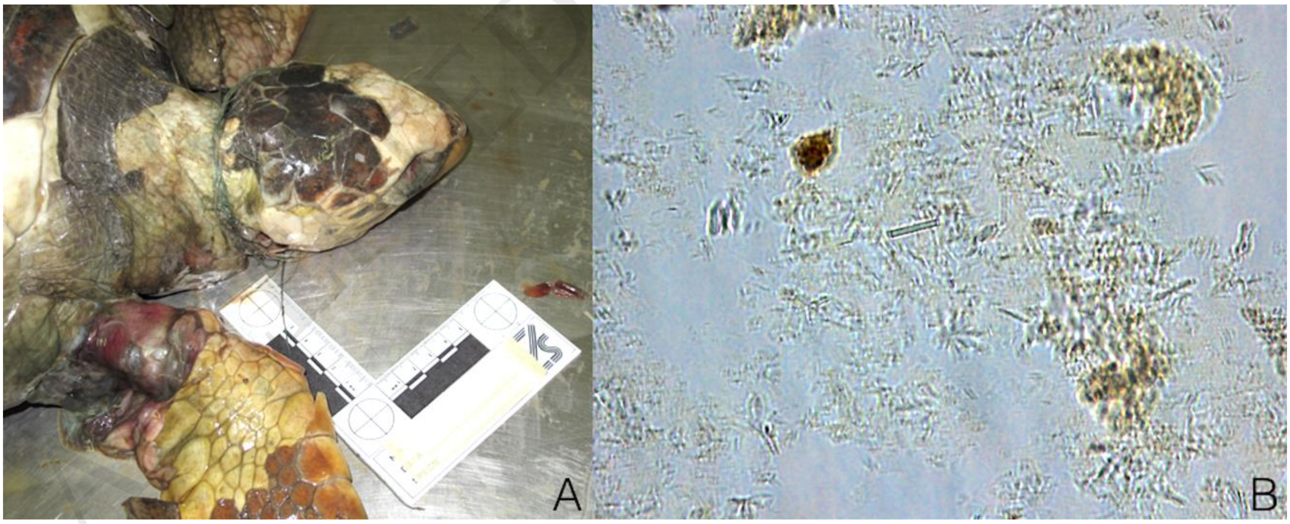

Fig. 2: diatoms in loggerhead turtle ID 10.

In fig. 2A lines around the neck of the loggerhead sea turtle (*Caretta caretta*) no. 10 are grossly evident; in fig. 2B diatoms found in the sea turtle's bone marrow are shown.

Table 1: Diatoms in cetaceans and sea turtles.

In this table findings and amount of diatoms found in 15 cm^3 (50 cm^3 in ID) 9 of bone marrow obtained from long bones of examined cetaceans and sea turtles are summarized. Information on the stranding site, the manner (MD) and the possible cause of death established following 2018-ICD-CM and aetiology and the carcass' preservation condition (CC).

MD	ID	Species	Stranding site	Cause of death	Etiology	CC	No. diatoms
NATURAL	1	*Stenella coeruleoalba*	Località Spiaggia d'oro (IM)	Infection complicated by chronic encephalitis with staphylococcal brain abscesses	Virus - DMV	2	0
	2	*Stenella coeruleoalba*	Prino (IM)	Systemic infection	Virus - DMV	3	0
	3	*Stenella coeruleoalba*	Genova	Infection complicated by encephalopathy with spongiosis and demyelination	Virus - DMV	3	>1000
	4	*Stenella coeruleoalba*	Savona	Infection complicated by chronic meningo-encephalitis and toxoplasmosis	Virus - DMV	2	0
	5	*Stenella coeruleoalba*	Bordighera (IM)	Toxoplasmosis complicated by chronic meningo-encephalitis	Protozoa - Toxoplasma gondii	3	0
	6	*Stenella coeruleoalba*	Imperia	Toxoplasmosis complicated by chronic meningo-encephalitis	Protozoa - Toxoplasma gondii	2	1
	7	*Stenella coeruleoalba*	Alassio (IM)	Bronco-pulmonary aspergillosis complicated by obstructive tracheitis and bronchitis	Mycotic - Aspergillus spp.	2	28
	8	*Stenella coeruleoalba*	Ventimiglia (IM)	Severe cardiomiopathy	Degenerative	3	0
AC	9	*Tursiops truncatus*	Lido di Volano (FE)	Drowning	Fishery interaction: possible entanglement in fishing net	3	>1000

C I D E N T A L	10	*Caretta caretta*	Riccione (FC)	Drowning	Fishery interaction - entanglement in fish-ing line	2	>30
	11	*Caretta caretta*	Pesaro	Drowning	Fishery interaction - entanglement in fish-ing line	3	>30
	12	*Caretta caretta*	Riccione (FC)	Traumatic injures	Rotating propeller	3	0
	13	*Caretta caretta*	Porto Sant'Elpidio (FM)	Traumatic injures	Rotating propeller	3	0
	14	*Caretta caretta*	Lido delle Nazioni, Comacchio (FE)	Traumatic injures	Rotating propeller	3	0

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